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A Modified Microtiter Plate Method for Biochemical Characterization of *Listeria* spp.

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ABSTRACT

A microtiter plate format previously reported for the biochemical characterization of microorganisms was modified by adding agar to all of the different test media instead of using a combination of liquid and solid media. This modification, termed the Modified Microtiter Plate procedure (MMP), offered the same advantages of the original method (labor saving, inexpensive, and custom designed for special needs) as well as having the added advantages of longer term storage prior to use and ease of handling. In this study, 60 bacterial isolates (both *Listeria* suspects and known *Listeria* cultures) were biotyped using the MMP protocol and compared to results obtained using the classical tube-based US-FDA recommended protocol as the standard identification method and the BBL-Minitek system.

The development of rapid and automated methods to identify microorganisms is an important aspect of food microbiology (*2*). Clinical, food, and environmental monitoring laboratories which handle very large sample numbers and need rapid results rely on the use of specialized and often expensive equipment and kits to conduct microbial testing to achieve these goals. Researchers wishing to identify large numbers of isolates and to obtain a more detailed biochemical characterization of these organisms may not be as concerned with obtaining the most rapid result possible. The purpose of this research was to evaluate a modified microtiter plate procedure (MMP) for characterizing *Listeria* spp.

MATERIALS AND METHODS

Characterized Listeria *organisms*

Known *Listeria* isolates were obtained from the culture collection at the Roman L. Hruska U.S. Meat Animal Research Center, USDA-ARS, Clay Center, NE (MARC). Isolates were held as glycerol suspensions at -20°C and grown in tryptic soy broth + 0.5% yeast extract (TSBYE, Difco, Detroit, MI) at 37°C prior to use.

Isolation of Listeria *suspects*

Suspect *Listeria* isolates were obtained from samples of manure from 224 cattle held in groups of 10 in feedlots at MARC. Ten grams of feces was taken from each animal within a pen and combined into a 100-g composite sample. The composite sample was added to 900 ml of secondary enrichment broth (*7*) and incubated for 24 h at 35°C. Secondary enrichment broth is composed of proteose peptone, 5 g; tryptone, 5 g; beef extract, 5 g; yeast extract, 5 g; NaCl, 20 g; KH_2P0_4, 1.4 g; K_2HP0_4, 12 g; esculin, 1 g; nalidixic acid (2% in 0.1 N NaOH), 1 ml; and distilled water, 1 L. Following autoclaving (121°C/15 min), 1 ml of 2.5% (w/v) filter sterilized acriflavin was added per liter of medium. Each composite enrichment was then streaked to LPM agar (BBL, Cockeysville, MD) or *Listeria* Selective Agar (Oxford formulation; Oxoid, Columbia, MD) and incubated at 37°C for 18-24 h. Suspect *Listeria* isolates were obtained by picking colonies which were catalase positive and either blue-green when viewed under the Henry's oblique lighting technique (from LPM agar) or typically black-brown colonies surrounded by a black halo from esculin hydrolysis (from Oxford agar). Those isolates which were gram-positive short rods were then streaked to TSBYE agar plates and incubated overnight (16-20 h) at 37°C to obtain isolated colonies.

Preparation of modified microtiter test plates (MMP)

The biochemical tests chosen for use in characterizing *Listeria* suspects were those reported in the procedure of Lovett (*6*). All formulae were the same except that 1.5% (w/v) agar was added to each medium and held at 50°C after sterilization. An 8-channel multi pipettor was used to add 100 µl (unless noted) of the media to the wells of sterile 96-well polystyrene microtiter plates, with covers (Falcon plastics, Becton Dickinson Labware; Lincoln Park, NJ). The media were added as follows: column 1, sheep blood agar (200 µl); column 2, urea agar; column 3, nitrate agar; columns 4 and 5, MRVP agar; column 6, triple sugar iron agar (200 µl); columns 7-12, glucose, D-xylose, L-rhamnose, manntiol, esculin, and maltose agars, respectively. The carbohydrate test agars were all purple broth base agar supplemented with 0.5% (w/v) of the test sugar. The media were added very carefully in order to avoid bubble formation. Up to 12 MMP were prepared simultaneously and allowed to solidify for 15 min. The plates were then wrapped in plastic film and held at 5°C until used.

Inoculation of MMP

Suspect colonies from TSBYE plates were suspended in 3 ml of physiological saline to a turbidity equivalent to a McFarland no. 3 standard or approximately $9x10^8$ CFU/ml. Each isolate suspension was used to inoculate one entire row of the MMP as described below. Twenty µl of the suspension was used to inoculate wells of columns 2-5 and 7-12 of the MMP plate. Wells of columns 1 (sheep blood agar) and 6 (triple sugar iron agar) were stab inoculated with an inoculating needle. The inoculated MMP plates

[1]*Mention of trade names, proprietary products, or specific equipment does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.*

were placed in a gallon capacity zip seal plastic bag containing a moistened paper towel to maintain a humid environment and incubated at 37°C.

Following 24-36 h incubation, the reactions were developed and read according to the US-FDA protocol for *Listeria* testing (*6*) by adding reagents directly to the appropriate test well. Weak β-hemolysis was evident as a clear, narrow zone in the agar extending from top to bottom of the stab. Strong β-hemolysis, as exhibited by *Listeria ivanovii*, was evident as a nearly complete clearing of the well. Test reaction results were easier to read using a microtiter plate mirror stand. All reactions were compared to rows inoculated with sterile saline.

Motility tests and CAMP reactions

These characteristics were determined using the standard methods described by Lovett (*6*) with the same inocula described above. Motility stabs were incubated at 23°C for up to 5 d.

Comparison to other identification methods

The same suspect isolates and known *Listeria* cultures were characterized by the classical identification scheme described by Lovett (*6*) as well as the Mini-Tek system (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD). With the Mini-Tek system, all standard *Listeria* diagnostic tests (*6*) were performed except the methyl red, triple sugar iron, hemolysis, CAMP, and motility reactions.

Listeria *identification computer program*

A BASIC computer program (written by Mr. M. W. Kattan, University of Arkansas, Dept. of Food Science, Fayetteville, AR) was based on the accepted identifying characteristics of all of the species in the genus *Listeria* (*6,9*). The program considers results entered as positive, negative, and variable and then prints out a profile sheet which analyzes the reactions characteristic for each species.

RESULTS

Twenty known *Listeria* isolates were tested using the MMP method and compared to results obtained using the FDA and Mini-Tek procedures (Table 1). Of these 20, there was complete agreement (100%) between the results obtained from the MMP procedure and the standard US-FDA procedure. The Mini-Tek procedure results were in complete agreement with the MMP results except for two *Listeria monocytogenes* isolates which were negative for the rhamnose test in the Mini-Tek battery.

Profiles of isolates taken from feedlot cattle manure were in complete agreement (100%) when tested by the MMP and US-FDA protocols. Thirty-nine *Listeria innocua* strains were isolated from composites from four feedlot pens. Twenty of these were found to be rhamnose negative and the remaining 19 were rhamnose positive when assayed by either the MMP or the US-FDA protocol. Three of the isolates found to be rhamnose positive by the MMP and US-FDA methods were found to be negative in the Mini-Tek profile.

The profile of an *L. monocytogenes* isolate from cattle manure was identical when tested by MMP, US-FDA, and Mini-Tek methods. This isolate was atypical of *L. monocytogenes* in that it was rhamnose negative. This isolate gave a positive reaction when analyzed in a dot blot EIA with a monoclonal antibody specific for *L. monocytogenes*, *L. innocua*, and *L. welshimeri* (*10*), thereby eliminating the possibility of it being , *L. seeligeri*. The isolation of rhamnose negative *L. innocua* strains from manure is not an unusual finding since only 11-89% of isolates demonstrate the ability to ferment rhamnose according to the characteristics reported by Seeliger and Jones (*9*). All of these isolates were found to react with a monoclonal antibody capable of reacting with *L. innocua* when assayed in a dot blot EIA (*10*).

The formation of acid from maltose in the MMP protocol was weak after 24 h of incubation but was fully developed (bright yellow color) after 36 h at 37°C. The carbohydrate test wells were overlayed with sterile mineral oil in one trial to test the effect of a microaerophilic environment on the speed of acid development. All reactions occurred in the same time frame as without the overlay with the exception of *L. ivanovii* which fermented xylose in less than 24 h when overlayed with mineral oil (data not shown). Based on this trial, the use of mineral oil overlays was omitted. All other reactions in the MMP protocol were clearly readable 24 h postinoculation. If the inoculated plates were held at 37°C for 3 to 4 d, the purple broth base agar wells began to fade in color; therefore, reading the plates

TABLE 1. *Comparison of different identification protocols for characterizing* Listeria *spp. ([+] = complete agreement in individual test results between protocols, [-] = variation in results obtained between protocols).*

Isolate	Protocol MMP[1]	FDA[2]	Mini-Tek[3]
L. monocytogenes			
ATCC 15313	+	+	+
Brie 1	+	+	+
#171	+	+	+
CAP Unk.	+	+	+
F1057	+	+	+
F1109	+	+	+
F2379	+	+	+
LCDC 81-861	+	+	-[4]
Murray B	+	+	+
Scott A	+	+	+
V7	+	+	+
V37CE	+	+	+[4]
L. grayi ATCC 19120	+	+	+
L. innocua LA-1	+	+	+
L. innocua ATCC 33090	+	+	+
L. ivanovii KC1714	+	+	+
L. seeligeri SE-31	+	+	+
L. seeligeri LA-15	+	+	+
L. welshimeri ATCC 35897	+	+	+
Feedlot cattle isolates			
L. innocua (Rhamnose neg.)[5]	+	+	+
L. innocua (Rhamnose pos.)[6]	+	+	-[4]
L. monocytogenes	+	+	+

[1]MMP, Modified Microtiter Plate protocol.
[2]FDA, Standard US-FDA *Listeria* protocol.
[3]Mini-Tek, used according to manufacturer's instructions.
[4]Gave negative rhamnose reactions when compared to the other two protocols.
[5]Twenty of 39 *L. innocua* isolates were rhamnose negative.
[6]Nineteen of 39 *L. innocua* isolates were rhamnose positive.

LISTERIA IDENTIFICATION PROGRAM

UNKNOWN CODE: L. monocytogenes Brie 1
ENRICHMENT: none
MEDIUM: TSBYE
SAMPLE NAME: cheese isolate
NUMBER OF TESTS ENTERED IS: 17

Gram stain	+
Sheep blood stab	+
Catalase	+
Urea	-
NO_3-Red	-
Motility	+
MR	+
VP	+
TSI	+
Glucose	+
Xylose	-
Rhamnose	+
Mannitol	-
Esculin	+
Maltose	+
S. aureus - CAMP	+
R. equi - CAMP	-

STANDARD	*MATCHES*	*MATCHES IF V +*	*MATCHES IF V -*
L. monocytogenes	17	17	17
L. grayi	13	13	13
L. murrayi	12	12	12
L. ivanovii	15	15	15
L. innocua	14	14	14
L. welshimeri	13	13	13
L. seeligeri	15	15	15
L. denitricians (Jonesia denitrificans)	11	11	11

Figure 1. *Example printout of the* Listeria *Identification Program written in BASIC computer language. See text for explanation of result entries. ([+] = positive, [-] = negative, [v] = variable).*

between 24 to 36 h is very important to assure accurate results.

An unidentified, non-*Listeria* isolate (gram-positive cocci, nitrate reductase positive) was put through the MMP protocol to determine if the nitrate agar would yield a positive result after just 24 h incubation. The nitrate test was clearly positive after 24 h incubation at 37°C upon adding the developing reagents.

The *Listeria* identification computer program greatly aided in compiling data to make a biotype profile of each isolate. A typical printout from this program is shown in Fig. 1. Results from diagnostic reactions are entered as positive (+), negative (-), or a variable (v). For example, a positive reaction for the Gram stain is gram-positive, short rods. Any other reaction or morphology would be entered as a negative reaction. In the case of the triple sugar iron reaction, a positive reaction is acid production throughout the stab with no evidence of gas formation.

DISCUSSION

Results obtained using the MMP were identical to those using the standard tube-based *Listeria* identification protocol of the US-FDA. The basic method developed by Fung and Hartman (*3,4*) to characterize large numbers of microbial isolates is rapid, inexpensive, and could be custom designed by the researcher for his/her own particular needs. Modifying this basic procedure by adding agar to all the test media allows greater ease of handling and longer storage of prepared plates. The possibility of liquid media spilling or cross-contamination between wells is eliminated.

The results from the MMP can be read after 24-36 h of incubation compared to up to 5-7 d recommended by the US-FDA standard procedure for *Listeria* spp. identification. The CAMP test reactions and the motility stabs took 24 and 96 h to develop, respectively. However, the use of the microscopic tumbling motility test would lessen the total time needed to perform motility testing and a complete characterization. After completion of testing, the plates were autoclaved and discarded, requiring no further handling.

All media and test reagents used are standard to most microbiology labs. Since large numbers of MMP plates can be prepared at once and held at 5°C, it would be possible to perform quality control checks on entire batches or lots of MMP plates if this information were needed by the quality assurance microbiologist. The MMP method eliminated tube manipulations and multiple loop sterilizations between each inoculation so that inoculation can be done in a fraction of the time it takes to inoculate the same number of tubes.

Use of MMP methodology and interpretation of results with the Listeria ID computer software was an efficient method to identify and characterize large numbers of suspect *Listeria* isolates. Analysis of results was performed easily by technical personnel who had no prior knowledge of computer use. It is possible to use individual plate well strips (Removawell strips, Dynatech Laboratories, MacClean, VA) instead of solid 96-well plates, thereby allowing identification of one isolate at a time. On the other hand, for analyzing large numbers of isolates, such as a collection of isolates collected over the course of an extended study, the use of a multi-point inoculator with a "master plate" as originally recommended by Fung and Hartman (*4*) for mass inoculation of 96-well plates could be adapted for use with the MMP protocol.

While this method was not as rapid as some other biochemical methods previously reported for the identification of *L. monocytogenes* and *Listeria* spp. (*1,5,8*), it provided a means to obtain a detailed biochemical profile to speciate *Listeria* isolates and compare the results to those recommended by the US-FDA protocol. This method did not require any special kits, reagents, or equipment not found in most microbiology laboratories.

The MMP protocol offers an easy and relatively rapid miniaturized method to characterize and identify *Listeria* isolates obtained from standard *Listeria* enrichment procedures. This same modified protocol has the potential to be customized for other groups of microorganisms of importance to the food microbiologist.

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